

## THE PRODUCTION OF BACITRACIN SYNTHETASE BY *BACILLUS LICHENIFORMIS* ATCC 10716

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### 1. Introduction

Bacitracin synthetase is an enzyme complex [1,2] composed of three complementary fractions called A, B and C [3] (see fig.1). Phosphopantetheine which is covalently bound to the fractions [2,3,5] is probably involved in the biosynthesis of bacitracin. Similar to gramicidin S and tyrocidine [6] its mechanism of synthesis has been described as a thiotemplate mechanism [7]. In the present work the bacitracin synthetase

production has been investigated. When *Bacillus licheniformis* was grown in the defined medium M 20, bacitracin was produced during growth [8]. It was shown that the three enzymes A, B and C of bacitracin synthetase occurred simultaneously for a short time early in the growth phase. The bacitracin synthetase harvested at this stage was able to produce bacitracin for more than one hour and at least 38% of the radioactive substrate amino acid could be incorporated into bacitracin. Later in the growth phase, bacitracin synthetase seemed to associate with the membranes or cell walls. Furthermore, it was shown that the level of bacitracin synthetase which was found in the cells did not correspond to the titer of bacitracin in different media.

### 2. Methods and materials

#### 2.1. Growth of *B. licheniformis*

Spores (about  $2 \times 10^7$ ) of *B. licheniformis* ATCC 10716 were suspended in 200 ml medium M 20 [8]. pH was adjusted to 6.0 with NaOH. After incubation in a New Brunswick rotary shaker (16 h, 37°C) the culture was used to inoculate 8 litres of medium M 20 (pH 6) in a New Brunswick fermentor.

The cells were grown at 37°C with stirring (500 rev/min and aeration (8 litres air/min). The cells were harvested early in the growth phase  $A_{650\text{nm}}$  1.2 in a 1 cm cell in Spectronic 20 spectrophotometer) and washed with 0.04 M potassium phosphate buffer, pH 7.2, containing 2 mM  $\text{MgSO}_4$  and then stored at -20°C.

#### 2.2. Bacitracin synthetase

Frozen cells were thawed and lysed with the aid of

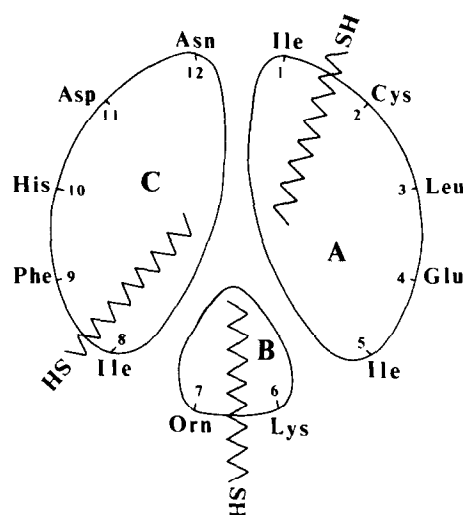


Fig.1. Schematic representation of bacitracin synthetase. A, B, and C represent the three complementary enzymes of bacitracin synthetase. The activation sites of the constituent amino acids (numbered 1–12 from the N-terminal end) in bacitracin, are indicated on the figure. The zig-zag line illustrates covalently linked phosphopantetheine.

lysozyme, and a 43–49% satd.  $(\text{NH}_4)_2\text{SO}_4$  fraction was prepared as previously described [2].

### 2.3. Fractionation of bacitracin synthetase

Bacitracin synthetase was fractionated into three complementary enzymes A, B and C on a 3, 3'-diaminodipropylamine–Sephadex column [3,7].

### 2.4. Estimation of bacitracin synthesis

The reaction mixture was modified from that previously used [2]. It contained 2  $\mu\text{mol}$  ATP, 1  $\mu\text{mol}$   $\text{MnCl}_2$  (the pH of the solution of ATP and  $\text{MnCl}_2$  was adjusted to 7.4 with KOH), 5  $\mu\text{mol}$  potassium phosphate buffer, pH 7.4, 0.1  $\mu\text{mol}$  dithiothreitol, 0.02  $\mu\text{mol}$  of all the L-forms of the amino acids in bacitracin except one which was  $^{14}\text{C}$ -labelled (0.1  $\mu\text{Ci}$ , 0.01  $\mu\text{mol}$ ) and enzyme solution in a final volume of 0.1 ml. The mixture was incubated for 30 min at 37°C and bacitracin was isolated and identified as described [2].

### 2.5. Estimation of ATP– $[\text{}^{32}\text{P}]\text{PP}_i$ exchange reaction

The incubations were carried out at 37°C for 30 min and the exchange reaction was determined as described [2].

### 2.6. Labelled substances and measurement of radioactivity

L-[U- $^{14}\text{C}$ ]Isoleucine and  $\text{Na}_4^{32}\text{P}_2\text{O}_7$  were obtained from New England Nuclear Corp., Boston and from The Radiochemical Centre, Amersham. For counting a Packard Tri-Carb Liquid Scintillation counter or a Friesche and Hoepfner gas-flow counter was used.

## 3. Results

### 3.1. The time of harvest

In the medium M 20 bacitracin was produced during growth [8]. The bacitracin synthetase activity occurred early in the growth phase (fig.2). An enzyme fraction

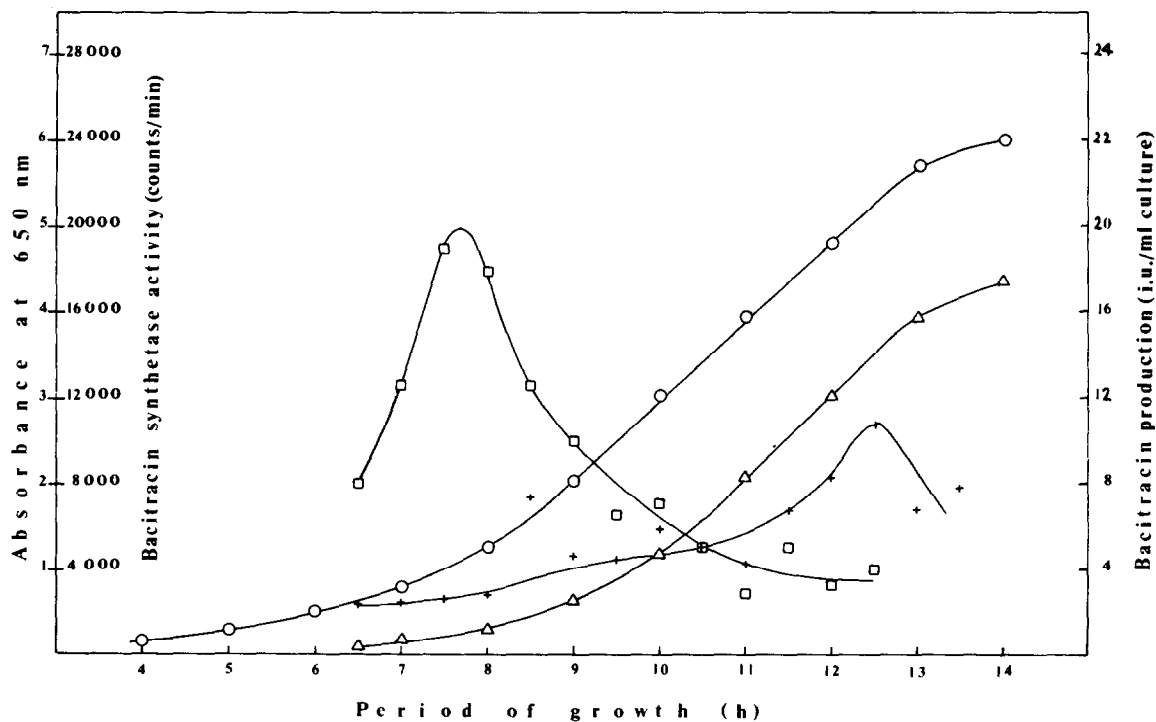


Fig. 2. Bacitracin production and bacitracin synthetase activity in relation to bacterial growth in medium M 20. The culture was grown as described in the experimental section. At intervals 20 ml aliquots were withdrawn. The absorbance (○) of the culture was measured, the bacitracin content (△) was determined microbiologically and the bacitracin synthesizing activity in the 55% satd.  $(\text{NH}_4)_2\text{SO}_4$  fraction (□) and in the pellet fraction (+) were assayed as described. The total amount of radioactivity in each incubation mixture was 110 000 cpm.

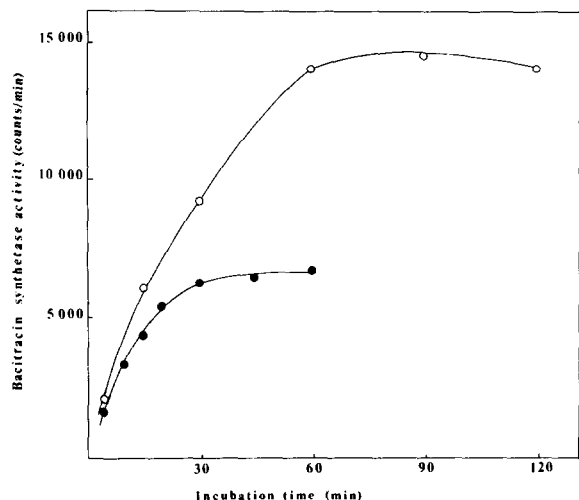


Fig.3. Bacitracin synthesizing activities in different enzyme preparates. Bacitracin synthesis was measured as described in 43–49% satd.  $(\text{NH}_4)_2\text{SO}_4$  fractions (10  $\mu\text{l}$ ) isolated from cells (20 ml), grown as previously described [2] (●), at  $A_{650}$  3.0 and from cells grown in medium M 20 (○), at  $A_{650}$  1.2. The total amount of radioactivity in each incubation mixture was 104 000 cpm.

prepared at this stage of growth seemed to be free (or partially free) of proteolytical activities since the addition of paramethylsulfonylfluorid, an inhibitor of proteolytical activities, did not stimulate bacitracin production (results not shown). The enzyme fraction was able to produce bacitracin for more than one hour. Of the labelled amino acid (L-isoleucine) 12% was incorporated into bacitracin. When the concentration of L-isoleucine was 0.01 mM, 38% of it was incorporated into bacitracin. In fig.3 the synthetase activity, as a function of time, of this enzyme preparate and that previously used [2] are compared. A three-fold increase of the time of synthesis was obtained by the new procedure.

Bacitracin synthetase occurs for only a short period during growth (fig.2). The onset of synthesis of the individual enzymes of the enzyme complex seems to occur simultaneously. This was shown by isolating bacitracin synthetase at different stages of growth. The presence of the enzymes A, B and C was shown by measuring the  $[^{32}\text{P}]\text{PP}_i$ -ATP exchange reaction in the presence of specific amino acids [2] after alkyl-Sepharose chromatography [3] (results not shown).

Table 1  
Effect of manganese on the production of bacitracin and bacitracin synthetase

$\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (mg/litre)	Bacitracin produced during 16 h (IU/ml)	Level of bacitracin synthetase (cpm)
0	1.5	12 500
0.2	15	19 000
10	40	18 500

The culture was grown as described in medium M 20 (which contains 10 mg  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ /litre and at reduced manganese concentrations. Bacitracin was assayed microbiologically and the maximum level of bacitracin synthetase was measured as described under fig.2

### 3.2. Dependence of manganese

The production of bacitracin in the medium was strongly dependent on the concentration of manganese. When zero, 0.2 or 10 mg  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ /litre medium was used, 1.5, 15 and 40 IU bacitracin/ml were obtained (see table 1). However, this does not correspond to the production of bacitracin synthetase. In the three experiments the level of cell-free bacitracin synthetase activity was almost the same.

### 3.3. Pellet-bonded bacitracin synthetase

The cell free (soluble) bacitracin synthetase activity was lost during growth ((□—□) fig.2). The activities of the complementary enzymes A, B and C disappeared simultaneously as shown by the  $[^{32}\text{P}]\text{PP}_i$ -ATP exchange reaction [2] (results not shown). However, in vivo bacitracin production still occurred later in the growth period. At this stage of growth the bacitracin synthetase activity seemed to be tightly associated with the cell fragments ((+—+) fig.2). Repeated lysozyme treatment did not release the synthetase into solution. The drop in soluble plus pellet-bonded activities (not drawn on fig.2) between the two activity peaks on fig.2 is probably due to proteolytical activities and/or the unsuitable incubation conditions at lower synthetase concentrations.

## 4. Discussion

The bacitracin production seems not to reflect the

level of bacitracin synthetase in different media. A control mechanism of bacitracin synthetase is probably involved.

It has been reported that peptide synthetases including bacitracin synthetase, only appear in the late growth phase [2,6]. The rapid destruction or inactivation of the enzyme complexes, after having reached their maximum activity peak is probably due to the presence of proteolytical enzymes [9] which appear at this time of growth in most bacilli. In the medium M 20 bacitracin was produced during growth [8] and bacitracin synthetase was isolated at an early stage. As far as we know, this is the first example of a peptide antibiotic synthetase which is isolated in the early growth phase. The absence of proteolytical activities probably made it possible to obtain a prolonged bacitracin production in vitro. For further studies on the biosynthesis of bacitracin this condition for cell harvest will be preferred.

The detection of bacitracin synthetase early in the growth phase and the formation of bacitracin during growth indicate a function of bacitracin during cell multiplication and not in the sporulation process as reported for gramicidines and tyrocidines [10]. The isolation and characterization of a defect bacitracin synthetase of a zero-producer of bacitracin which sporulates normally [7,11] supports this view.

The soluble bacitracin becomes predominately cell bound later in the growth phase. This does not seem to have any negative effect on bacitracin formation, and may represent a natural immobilization of an enzyme complex which probably protects it against increasing proteolytical activities.

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